ARTICLE

Impact of immunosuppressive drugs on the therapeutic efficacy of *ex vivo* expanded human regulatory T cells

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ABSTRACT

Immunosuppressive drugs in clinical transplantation are necessary to inhibit the immune response to donor antigens. Although they are effective in controlling acute rejection, they do not prevent long-term transplant loss from chronic rejection. In addition, immunosuppressive drugs have adverse side effects, including increased rate of infections and malignancies. Adoptive cell therapy with human Tregs represents a promising strategy for the induction of transplantation tolerance. Phase I/II clinical trials in transplanted patients are already underway, involving the infusion of Tregs alongside concurrent immunosuppressive drugs. However, it remains to be determined whether the presence of immunosuppressive drugs negatively impacts Treg function and stability. We tested *in vitro* and *in vivo* the effects of tacrolimus, mycophenolate and methylprednisolone (major ISDs used in transplantation) on *ex vivo* expanded, rapamycin-treated human Tregs. The *in vitro* results showed that these drugs had no effect on phenotype, function and stability of Tregs, although tacrolimus affected the expression of chemokine receptors and IL-10 production. However, viability and proliferative capacity were reduced in a dose-dependent manner by all the three drugs. The *in vivo* experiments using a humanized mouse model confirmed the *in vitro* results. However, treatment of mice with only rapamycin maintained the viability, function and proliferative ability of adoptively transferred Tregs. Taken together, our results suggest that the key functions of *ex vivo* expanded Tregs are not affected by a concurrent immunosuppressive therapy. However, the choice of the drug combination and their timing and dosing should be considered as an essential component to induce and maintain tolerance by Treg.

Introduction

Naturally occurring regulatory T cells (Tregs) play a critical role in maintaining peripheral tolerance to self-antigens and controlling autoimmune disease. They are also important to limit immune responses to foreign antigens such as alloantigens. As shown in experimental models, adoptive transfer of Tregs can ameliorate autoimmune diseases, graft-*versus*-host disease (GvHD) and also prevent solid

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organ transplant rejection.[1] More recently, we and others have demonstrated that adoptively transferred human Tregs can protect *in vivo* from human skin, vessel and islet transplant rejections.[1-4]

Adoptive cell therapy with human Tregs for the treatment of autoimmune diseases and for the induction of transplantation tolerance represents a promising strategy. Indeed, clinical trials using this approach have already demonstrated that Tregs are safe in the treatment of GvHD and Type 1 diabetes.[5]

We have recently identified a clinically applicable protocol for the expansion of human immunomagnetic bead-separated $CD4^+CD25^+$ Tregs.[6] We have shown that the expansion of Tregs using rapamycin provides a new and refined approach for large-scale generation of functionally potent and phenotypically stable regulatory T cells, rendering them safe for clinical use in the settings of inflammatory diseases.[6,7]

Our group is currently involved in a multi-center phase I/II study to investigate the safety of infusing *ex vivo* expanded Tregs in solid organ transplantation (the ONE Study, funded by the European Union FP7 program). In this trial, Tregs expanded *in vitro* are being injected in kidney transplant patients receiving concurrent immunosuppressive drugs (ISDs).

Immunosuppressive drugs in clinical transplantation are necessary to prevent acute graft rejection. Various compounds have been selected and applied according to their ability to control lymphocyte activation.[8] However, despite the use of ISDs most of the transplants fail within ten years due to chronic allograft dysfunction.[9] In addition, ISDs are linked to morbidity and mortality.[8,10]

Conventional immunosuppressive therapy employs drugs such as calcineurin inhibitors, e.g. cyclosporine (CsA) or tacrolimus (TAC), as well as mycophenolate mofetil (MMF), an ester prodrug of the immunosuppressant mycophenolic acid (MPA) and corticosteroids such as methyl-prednisolone (mPr). More recently, many transplant centers have started to investigate rapamycin (RAPA)-based immunosuppression. The precise mechanism of action of these drugs is well characterized and different reports have described their effect on different cell subsets. ISDs are part of clinical protocols that accompany the adoptive transfer of Tregs. For example, the combination of TAC, MMF and mPr is part of the clinical protocol in the One Study. There is, therefore, the need to investigate the influence of ISDs on the viability, function and stability of *in vitro* expanded and adoptively transferred human Tregs.

Current evidence suggests that calcineurin inhibitors have markedly negative effects on circulating Tregs, mainly by interfering with IL-2 production and therefore affecting Treg function and survival.[11,12] Conversely, corticosteroids have been reported to increase Treg frequency and FOXP3 expression in patients with autoimmune diseases.[13,14] Finally, renal transplant recipients receiving MMF show significantly higher $CD4^+CD25^{hi}FOXP3^+$ Tregs compared to patients on other treatments.[12] Furthermore, studies on liver transplant patients with renal impairment on CsA and converted to MMF showed that the new treatment may reverse the negative effect of calcineurin inhibitors on Tregs.[15]

This study has focused on the consequences of using drugs commonly applied in the treatment of transplant patients, namely TAC, MPA and mPr, for the viability, function and stability of *ex vivo* expanded Tregs. Their effects were compared to the *in vivo* treatment with RAPA.

Methods

Cell isolation and separation

Peripheral blood mononuclear cells (PBMC) from healthy donors were obtained from anonymized human leukocyte cones supplied by the National Blood Transfusion Service (NHS blood and transplantation, Tooting, London, UK). Human studies were conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Guy's Hospital (reference 09/H0707/86). Informed consent was obtained from all healthy donors prior to enrolment into the study. PBMC were isolated by lymphocyte (PAA) density gradient centrifugation. $CD4^+CD25^+$T cells were purified by negative selection of $CD4^+$T cells followed by positive selection of $CD25^+$T cells using miniMACS $CD4^+CD25^+$T Regulatory Cell Isolation Kit (Miltenyi-Biotec) with a purity of 90-98%.

Human Treg-line generation

Human Tregs were expanded *ex vivo* with RAPA, as previously described.[6] In brief, human $CD4^+CD25^+$T cells were plated at $1x10^6$/mL in X-Vivo15 (Lonza), containing rapamycin (100nM; LC-Laboratories). Cells were activated with anti-CD3/CD28 beads (ratio bead:cell of 1:2; Invitrogen, UK). IL-2 (1000 IU/mL; Proleukin-Novartis) was added at day 4 post activation and replenished every two days. Cells were re-stimulated every 10-12 days and used after 36 days from the first activation (3 rounds of stimulation).

Antibodies for flow cytometry

Stained single-cell suspension were analyzed with: CD3-FITC (OKT4), CD4-FITC/APC (OKT4) from Sigma-Aldrich; CD62L-FITC (Greg-56; Invitrogen); CD25-PE (4E3), CD152-PE (14D3), CD127-PE-Cy7 (eBioRDR5), GITR-APC (eBioAITR), ICOS-PE-Cy7 (ISA-3), CD39-FITC (eBioA1), IL-10-APC (JES3-9D7), IFN-γ-FITC (4S.B3), IL-17-PE (eBio64DEC17) and FOXP3-eFluor 660 (PCH101) from eBioscience; CD27-PE (M-T271) and Integrin β7-PE-Cy5 (FIB504) from BD-Bioscience, UK; HLA-DR-APC (L243), CLA-FITC (HECA-452) and CCR4-PerCP-Cy5.5 (TG6/CCR4) from Biolegend. Appropriate isotype controls from mouse or rat were used. Prior to use, all antibodies were titrated using normal resting or activated PBMC to establish optimal staining dilutions.

Treg culture with immunosuppressants

TAC, MPA and mPr (Sigma-Aldrich) for the *in vitro* study were diluted in DMSO and used at different concentrations (see Results for details). To test their effect on Tregs, cells were activated with anti-CD3/CD28 beads (ratio 1:2), IL-2 (20 IU/mL) in X-Vivo[15] in presence of drugs. Cell viability was measured by LIVE/DEAD®kit. Proliferation was evaluated by cell-count, Ki67 staining, CFDA-SE and CellTrace™ Violet (CTV) proliferation kit.

Xenogeneic GvHD and in vivo stability assay

Balb-c $RAG^{-/-}\gamma c^{-/-}$ mice were used between 8-14 weeks of age. Mice were maintained under specific pathogen-free conditions and handled in accordance with the Institutional Committees on Animal Welfare of the United Kingdom Home-Office (Home-Office Animals Scientific Procedures Act, 1986; reference PPL 70/7302). In order to establish whether injected Tregs could maintain a stable phenotype in the presence of immunosuppressive regimen, a Xeno-GvHD model was induced by intravenous transfer of $1x10^7$ HLA-

A2- PBMCs depleted of CD25$^+$cells into Balb-c RAG$^{-/-}\gamma c^{-/-}$mice. Animals were monitored 3-times weekly for body weight, other GvHD symptoms (hunched back, fur loss, skin inflammation), and assessment of human CD45$^+$cell engraftment. Immunosuppressive treatment using drugs for infusion in transplantation therapy was administered by intra-peritoneal injection when animals were reconstituted with approximately 10% human CD45$^+$cells and daily during the next three days. ISDs were used as previously described: 16-18 TAC 2 mg/Kg (Prograf, Astellas), MPA 100 mg/Kg (CellCept, Roche), mPr 20 mg/Kg (Solu-Medrone, Pharmacia) and RAPA 300 µg/Kg (LC-Laboratories). HLA-A2+Tregs (1x10^7) were introduced into this environment after one day of drug administration. Spleen and lymph nodes were harvested three days after Treg transfer. Tregs were recovered by cell sorting on a 3-laser FACS-ARIA high-speed cell sorter (BD Biosciences).

Additional information is available in the *Online Supplementary Appendix*.

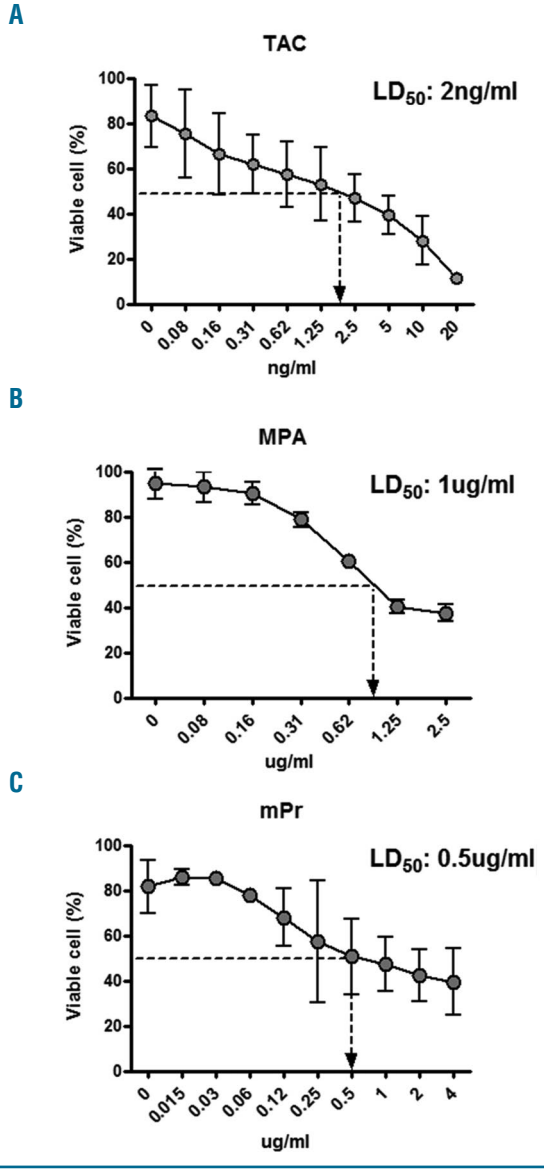

Figure 1. Dose-response curves to derive the concentration of immunosuppressant. *Ex vivo* expanded and rapamycin-treated Tregs were activated with anti-CD3/CD28 coated beads (bead:cell ratio = 1:2) and IL-2 (20 IU/mL) for three days in the presence of different concentration of drugs. (A-C) Concentration of TAC, MPA and methyl-prednisolone, respectively, leading to 50% of Treg viability. Data are combined from 4 independent experiments.

Results

Treg viability is affected by the presence of immunosuppressive drugs in vitro

To determine the direct effect of immunosuppressive drugs (ISDs) on *ex vivo* expanded Tregs, CD4$^+$CD25$^+$ T cells were purified by magnetic bead separation and cultured *in vitro* with anti-CD3/CD28 beads, IL-2 (100 U/mL) and rapamycin (RAPA), as previously published.[6] After 3 rounds of stimulation and 36 days in culture (GMP compliant protocol for the generation of Treg lines used in the

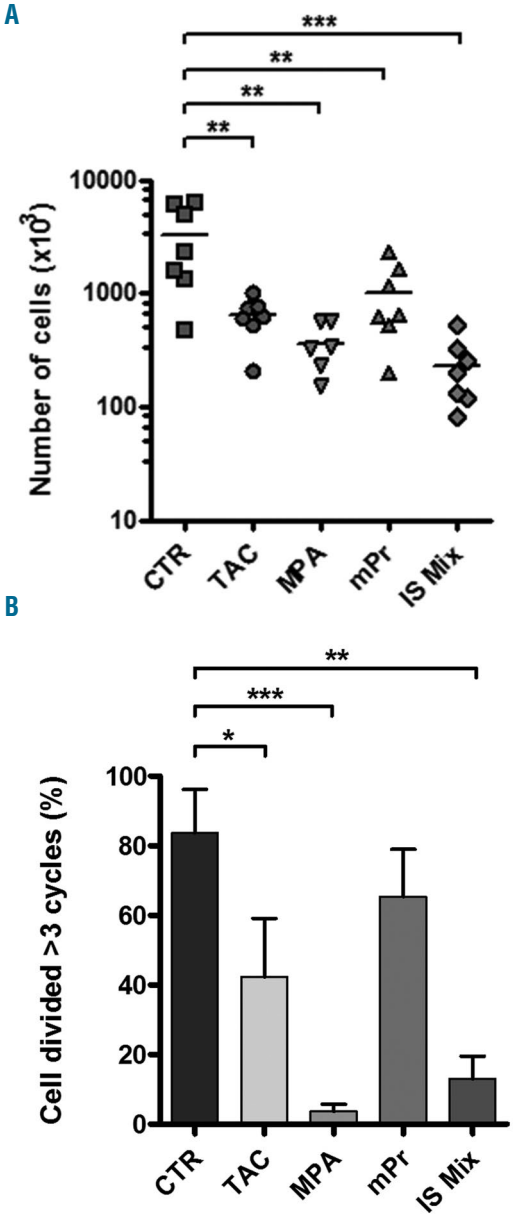

Figure 2. Proliferative capacity of Tregs in the presence of immunosuppressants. (A) Total number of *ex vivo* expanded and rapamycin-treated Tregs activated with anti-CD3/CD28 coated beads (bead:cell ratio = 1:2) and IL-2 (20 IU/mL) and cultured for five days with medium (CTR), single immunosuppressive agent at LD50 (TAC, MPA and mPR) or their combination (IS Mix). Data are combined from 7 independent experiments. (B) Percentages of Tregs determined by CTV dilution profile with more than three cycles of cell division cultured in the same conditions described above. Data are from 3 independent experiments and presented as mean ± SD. *P<0.05, **P<0.01; ***P<0.001.

One Study), Tregs were activated with anti-CD3/CD28 beads and 20 IU/mL of IL-2 in the presence of tacrolimus (TAC), mycophenolate (MPA) and methylprednisolone (mPr). Variability in pharmacokinetics and pharmacodynamics makes the therapeutic concentrations of these drugs indicative only in clinical practice.[19] Cross-sectional studies have revealed that trough levels may not correspond to the actual active concentrations on cells. Indeed, in blood, TAC is mainly associated with erythrocytes (>70%) and plasma proteins (>20%), and only a small fraction with lymphocytes (about 1%).[19] MPA is mainly bound to serum albumin (>97%)[20,21] and only the free fraction is thought to be responsible for the immunosuppressive effect.[20,21] Other data showed that renal function, serum albumin concentrations, hemoglobin levels and immunosuppressive co-medication, such as TAC, are factors contributing to the variability and time-dependent pharmacokinetics of drug treatments.[22,23] In this study, in which we aimed to translate *in vitro* the doses of ISDs used in clinical protocols, we tested a range of concentrations (TAC: 0.08-20 ng/mL; MPA: 0.08-2.5 μg/mL; mPr: 0.015-4 μg/mL) including both maximum and minimum therapeutic levels of ISDs, described in transplanted patients.[24-26] The doses leading to 50% of Treg viability (LD50) (2 ng/mL, 1 μg/mL and 0.5 μg/mL for TAC, MPA and mPr, respectively) were selected and used for the *in vitro* analysis (Figure 1A-C).

A significant reduction of cell proliferation was observed when Tregs were cultured for five days with either a single drug or the mixture of the three drugs (IS Mix), at the established LD50 (Figure 2A). Further analysis revealed that the number of cells going to division was affected mostly by TAC, MPA and by the IS Mix (Figure 2B).

The suppressive activities of in vitro *expanded Tregs is maintained in the presence of immunosuppressive drugs*

The maintenance of the suppressive ability and stability of Tregs are relevant to the success of Treg therapy. Therefore, we investigated the suppressive ability of Tregs in the presence of ISDs. Tregs and CFSE-labeled responder T cells (Teff) were activated and co-cultured in the presence of LD_{50} dose of TAC, MPA and mPr in isolation or in combination for five days. Results in Figure 3 show that ISDs did not have any negative effect on the suppressive capacity of Tregs and MPA, mPr and IS Mix, but rather strengthened it (1:10 ratio). To further investigate the effect on Tregs expanded with drugs, Treg lines were cultured for five days with the LD_{50} dose of ISDs, then washed and co-cultured with activated CFSE-labeled Teff. We observed that the suppressive ability was maintained even in these conditions (*Online Supplementary Figure S1*).

Immunosuppressive treatments have no effect on the expression of Treg functional molecules but affect chemokine receptors

Tregs have been described to express both surface and cytoplasmic molecules influencing their effector functions.[27] Furthermore, the expression of chemokine receptors by Tregs can confer to these cells specific homing abilities. Treg lines (previously expanded in the presence of rapamycin) were cultured for five days in the presence of TAC, MPA, and mPr in isolation or in combination. A complete analysis of these markers on Tregs revealed that while the expression of most of the molecules was not modified by the drug treatments (Figure 4A and B), the level of expression of CD25 (MFI) on Tregs was reduced by the treatment with TAC (Figure 4B and C).

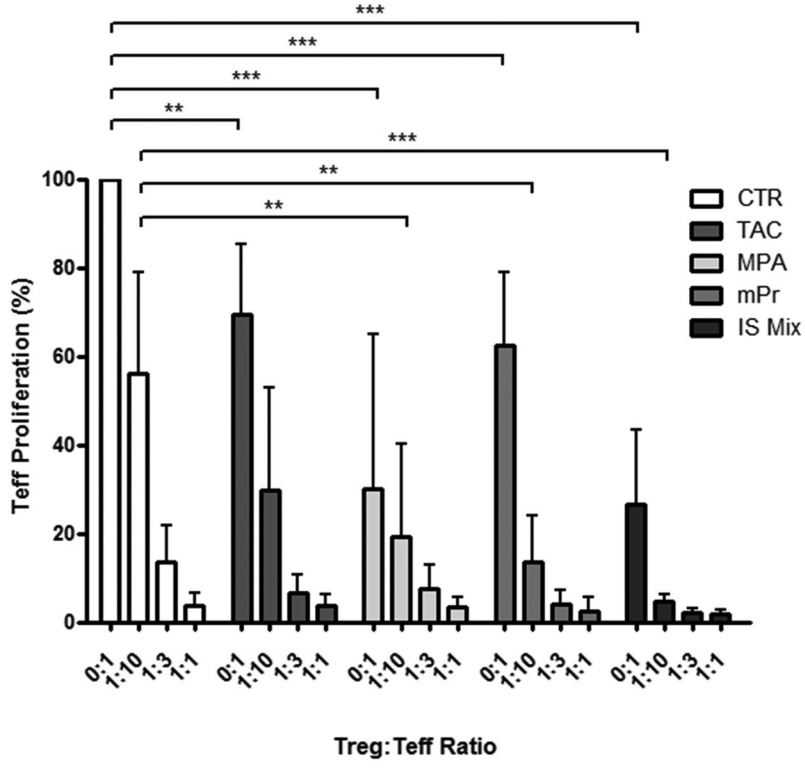

Figure 3. Effect of immunosuppressants on Treg suppressive ability. Proliferative responses (at 5 days) of Teff activated with anti-CD3/CD28 coated beads + IL-2 (20 IU/mL) and cultured alone or in co-culture with Tregs and different immunosuppressive agents. Proliferation of Teff, alone or in co-culture with Treg, was determined by CFSE dilution profile. Data are from 5 independent experiments. ***P*<0.01; ****P*<0.001.

Looking at the expression of chemokine receptors, we once again found that TAC reduced the expression of CCR4 and CLA (related to skin-homing) while the expression of integrin β7 (gut-homing) was up-regulated (Figure 4D).

ISDs maintain the stability of Tregs while IL-10 production is reduced by TAC

We and others have previously shown that RAPA can selectively favor the expansion of Tregs, increase IL-10 production and reduce the frequency of cells releasing pro-inflammatory cytokines such as IL-17 and IFN-γ.[6,28] To investigate the effect of drugs on the cytokine profile, Tregs were stimulated with anti-CD3/CD28 beads and IL-2 in the presence of TAC, MPA and mPr in isolation or in combination. After five days, culture supernatants were harvested and tested for the presence of cytokines. The amount of cytokine was normalized to the total number of live cells at the end of culture. TAC and mPr decreased the production of IL-17 and IFN-γ (Figure 5A and B) while the presence of TAC reduced IL-10 production by Tregs (Figure 5C) suggesting that TAC may affect the regulatory function of Tregs.

ISDs reduce the proliferative ability of adoptively transferred Tregs in a humanized mouse model

To extend the *in vitro* observations to an *in vivo* setting, we tested the effect of a concurrent immunosuppressive therapy on *ex vivo* expanded adoptively transferred RAPA-treated Treg preparations in a humanized mouse model. Immunodeficient mice (RAG2$^{-/-}$yc$^{-/-}$mice) were injected with human HLA-A2- PBMC (1x10^7) to create an inflammatory environment *in vivo*.[6] After 2-3 weeks, and long before the first signs of GvHD, one group of mice received daily intraperitoneal injection of IS Mix (TAC+mPr+MPA), for four days. Two other groups of mice received either the Treg permissive drug RAPA alone, 18 or the combination of RAPA and TAC, as many transplant centers have investigated sirolimus (RAPA)-based immunotherapies.[29] Another group of mice received saline solution only. Twenty-four hours after the first injection of immunosuppressive drugs, human HLA-A2$^+$ Tregs (1x10^7) were injected into the different groups of mice. After three additional days, mice were culled. Although in this setting mice did not show any sign of xeno-GvHD (e.g. ruffled fur, hunched posture), differences were observed in the size of the spleens between the four groups. Animals injected with drugs showed smaller spleens and a significant reduced number of total splenocytes compared to the control group (*Online Supplementary Figure S3*). The analysis of human/mouse chimerism in the spleens showed similar levels of human cell engraftment in all the groups of mice although the group receiving RAPA+TAC tended to be the lowest (Figure 6A). The average engraftment (±SD) in the control group was 51±22%: 54±16% in the IS Mix group, 45±8% in the RAPA group, and treated mice in the RAPA+TAC group 26±35% (Figure 6A). There was no difference in the percentage and the average number of adop-

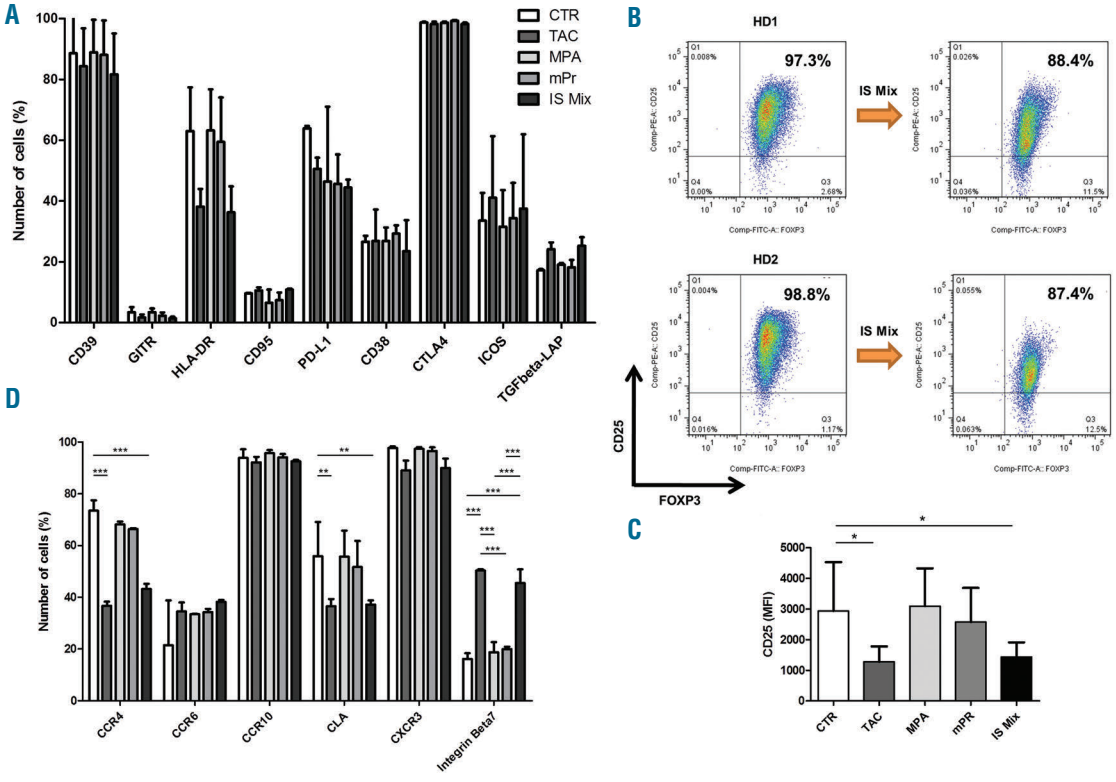

Figure 4. Phenotype of Tregs cultured with immunosuppressive agents. (A) Expression of Treg markers on cells treated for five days with medium (CTR), single immunosuppressive agent (TAC, MPA and mPR) or their combination (IS Mix). Data are from 5 independent experiments. (B) FOXP3 and CD25 expression on Tregs from 2 healthy donors (HD1, HD2). Data are representative of 9 different Treg preparations cultured five days with either medium (CTR) or with the combination of 3 drugs (IS Mix). (C) Cumulative data showing CD25 expression (MFI) on untreated and drug-treated Tregs. (D). Expression of a panel of chemokine receptors on Tregs cultured in the same conditions described above. Data are from 5 independent experiments. ***P*<0.01; ****P*<0.001.

tively transferred Tregs, detected within the total human CD45+ cells, by gating on HLA-A2 expression, between the four groups of mice, although a very high variability was observed (Figure 6B). The average engraftment of Tregs was 25±32% in the control group, 13±23% in the IS Mix group, 27±25% in the RAPA group, and 19±17% in the RAPA+TAC group (Figure 6B). These data suggest that the concurrent *in vivo* treatments with ISDs had no effect on Treg engraftment (Figure 6B).

Next, the number of dividing Tregs was investigated to understand whether any of the ISDs have affected the proliferation of Tregs *in vivo*. This was based on the possibility that ISDs, by altering the inflammatory environment and inhibiting, for example, IL-2 production, could have influenced the survival and proliferation of injected Tregs. As expected, we observed a significant difference in the number of proliferating non-Treg cells (HLA-A2-) between the control group (48±12%) and the three groups of mice treated with ISDs (IS Mix, 26±8%; RAPA, 22±11%; RAPA+TAC, 12±6%). However, the analysis of cycling Tregs (Ki67+ cells) showed that the IS Mix (4±1%) and RAPA+TAC (10±1%) treatments, but not RAPA alone, have decreased the number of proliferating cells in comparison to the control group (19±5%). Interestingly, the concurrent treatment with RAPA alone did not affect the division of Tregs (15±3%) (Figure 6C).

ISDs do not modify the phenotype, stability and function of adoptively transferred Tregs

The ability of adoptively transferred Tregs to maintain the phenotype, cytokine profile and suppressive function *in vivo* in an inflammatory environment was analyzed in the humanized mouse model described above. The analysis of the phenotype of the cells recovered from the spleens of the three groups of mice treated with the ISDs showed that the expression of FOXP3, CD25 and CTLA4 molecules were maintained compared to the control group (Figure 7A). The analysis of chemokine receptors showed only a marginal increase in the number of Integrin-β7+ Tregs in the drug-treated groups but the differences did not reach statistical significance (Figure 7B).

Next, in order to investigate whether the stability and function of Tregs were altered *in vivo* in the presence of immunosuppressive drugs and in a pro-inflammatory environment, we used cell sorting to purify human HLA-A2+ Tregs from splenocytes and tested them *in vitro* for their function. The cytokine profiles of sorted Tregs was analyzed by intracellular staining.[6] The results showed that IL-10, IL-17 and IFN-γ production were similar to that observed *in vitro* and the percentages of positive cells was identical between mice treated *in vivo* with all the different drugs or left untreated (Figure 7C). The suppressive capacity of *ex vivo* Tregs was tested *in vitro*. The results demonstrated that Tregs from either control or drug-treated mice maintained their suppressive ability (Figure 7D).

Discussion

Treg therapy is already in the clinic in the treatment of GvHD and Type I diabetes and has been shown to be safe.[5] At KCL we have started a clinical trial in renal transplant patients (the One Study) in which Tregs are separated from the peripheral blood of the patients who are going forward to receive kidney transplants and expanded *in vitro* in the presence of IL-2 and RAPA to obtain therapeutic numbers of highly pure Tregs. In our clinical trial, Tregs are infused into patients receiving additional therapies in the form of a combination of TAC, mPr and MMF. However, in many transplant centers, immunosuppressive regimens based on the use of sirolimus (RAPA) are under investigation.[5,29,30] In this study, we examined whether the co-administration of immunosuppressive drugs could

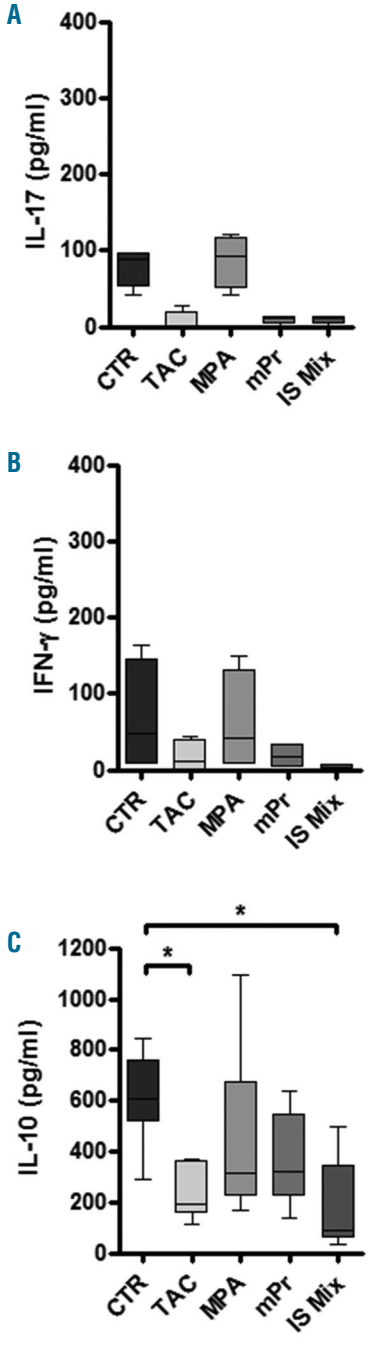

Figure 5. Effect of immunosuppressants on the production of cytokines by Tregs. Amount of IL-17 (A), IFN-γ (B) and IL-10 (C) in the supernatants from 5-day culture of Tregs activated with anti-CD3/CD28 coated beads (bead:cell ratio = 1:2) and IL-2 (20 IU/mL) and in the presence of single immunosuppressive agent (TAC, MPA and mPR) or their combination (IS Mix). Data are from 3 independent experiments. *P<0.05.

affect the phenotype, function and stability of *in vitro* expanded Tregs. We have demonstrated both *in vitro* and *in vivo* that the treatment with MMF, TAC and mPr, but not RAPA, decreased Treg viability and proliferation. In addition, none of the different ISDs altered Treg function and stability. However, we found that TAC had the biggest effect on Tregs, affecting not only their viability and proliferative capacity, but also the cytokine profile and the expression of some chemokine receptors.

The negative effect of TAC is not surprising. Tregs cannot produce IL-2, but their survival and suppressive activity depend on the exogenous supply of this cytokine.[31,32] TAC, by inhibiting calcineurin pathway, blocks IL-2 production from T cells making Tregs, and T effectors very susceptible to the inhibitory effect of this drug. This may explain, at least in part, the reduced size of the spleens and the lowered number of proliferating cells observed *in vivo* in the animals treated with either the IS Mix or RAPA+TAC. Furthermore, our *in vitro* results, revealed a direct effect of TAC on IL-2Rα (CD25) expression on Tregs. This inhibition, previously described with T lymphocytes,[33] may aggravate even more the already critical Treg survival during immunosuppressive drug administration. Although previous data suggested that the treatment of transplant recipients with low doses of TAC may be beneficial to Tregs,[34] there are many other studies providing evidence of the potentially harmful effect of calcineurin inhibitors on human Tregs.[35-37] Although, we have not found any evidence of a negative effect of TAC on the FOXP3 expression, function and stability of our Treg preparations, some studies in animal models reported that CsA treatment reduced Foxp3 expression in natural Tregs,[32,38] diminished the frequencies of $CD4^+CD25^+Foxp3^+$ T cells,[39] and failed to support the dif-

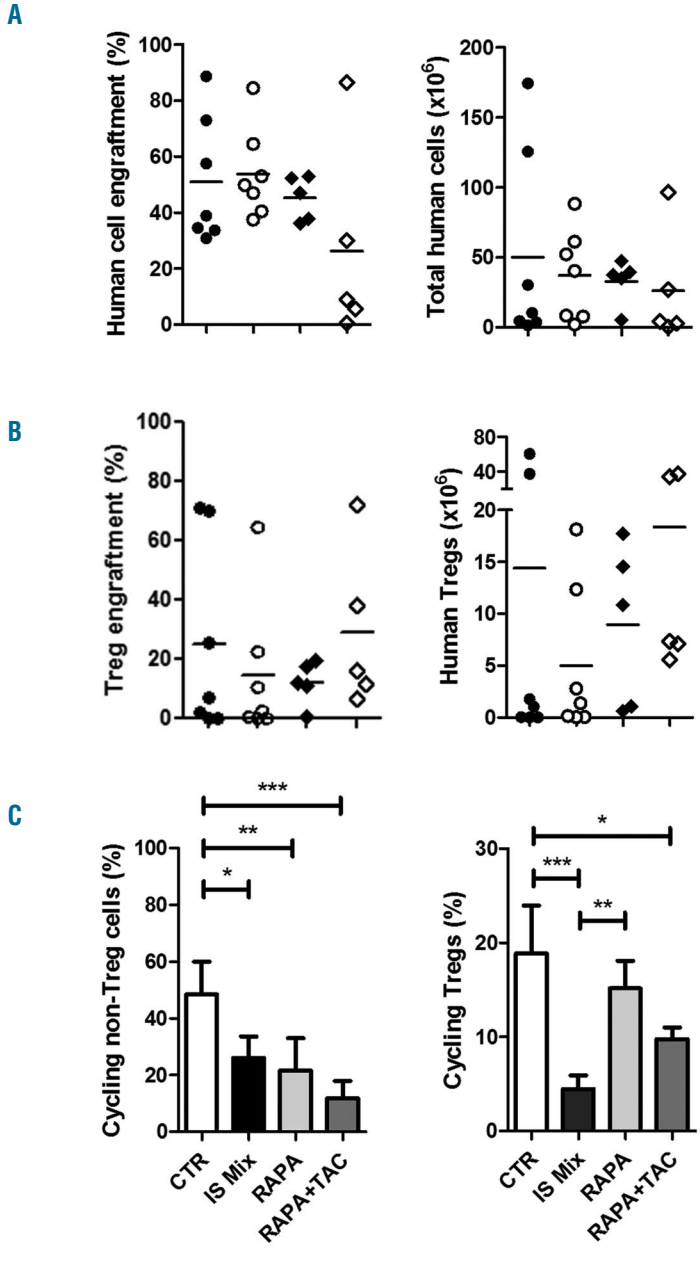

Figure 6. Effect of immunosuppressants on human cell engraftment in humanized mice. (A) Percentages of engraftment and absolute numbers of total human $CD45^+$ cells in spleen of mice receiving immunosuppressive treatment (IS Mix, RAPA and RATA+TAC) and controls (CTR). (B) Percentages of engraftment and absolute numbers of human Tregs ($HLA\text{-}A2^+$ cells) in spleen of mice receiving immunosuppressive treatment (IS Mix, RAPA and RATA+TAC) and controls (CTR). (C) Percentages of proliferating Teff (left) and Tregs (right) in the four groups calculated as Ki-67^+ cells. Data are representative of 4 independent experiments. *P<0.05; **P<0.01; ***P<0.001.

ferentiation of the highly suppressive $CD4^+CD25^+CD27^+$ subset upon alloantigen stimulation.[40] In addition, calcineurin inhibitors have been shown to affect Treg stability by directly interfering with NFAT signaling, and, as a consequence, its interaction with FOXP3.[37] In the experiment in which TAC was injected in mice together with RAPA, it was clear that TAC had a dominant effect on RAPA leading to a decrease in Treg viability and proliferation.

The detrimental effect observed with ISDs on Tregs might not be limited to TAC, but MPA could have also played a role *in vivo*, as shown *in vitro*. In literature, the effect of this drug on Tregs has not been extensively studied and the few results reported are controversial. MPA has been shown to inhibit inosine 5'-monophosphate dehydrogenase, a rate-limiting enzyme in the *de novo* synthesis of guanine nucleotides; thereby it affects DNA synthesis and cell replication of conventional T cells, as well as Tregs.[11] Our results confirmed this negative effect on cell replication *in vitro* and *in vivo* when administered as IS Mix cocktail. Our findings are supported by data in literature describing the influence of this drug on the expansion of antigen specific Tregs and establishing long-term tolerance; Wu *et al*. showed, in a murine model, that MMF administration significantly inhibited the expansion of OVA-specific $CD4^+CD25^+Foxp3^+$ Tregs after OVA immunization.[41] Similarly, Lim *et al*. showed that MPA adversely affected the therapeutic effectiveness of adoptively transferred Tregs, reducing the median survival time of allogenic skin transplants in recipient mice from 21 to 16 days.[17] On the other hand, some authors suggested that the administration of MPA have a positive role in Tregs. Zeiser *et al*. demonstrated that the concurrent administration of freshly isolated Tregs and MPA in a murine model of GvHD did not significantly inhibit either Treg expansion or suppressive ability and showed that the overall survival of the mice was extended.[32] Other reports suggested a role for this drug in the induction of a more tolerogenic environment. Indeed, Shibutani *et al*. showed that generation of Tregs in mice by intra-tracheal delivery

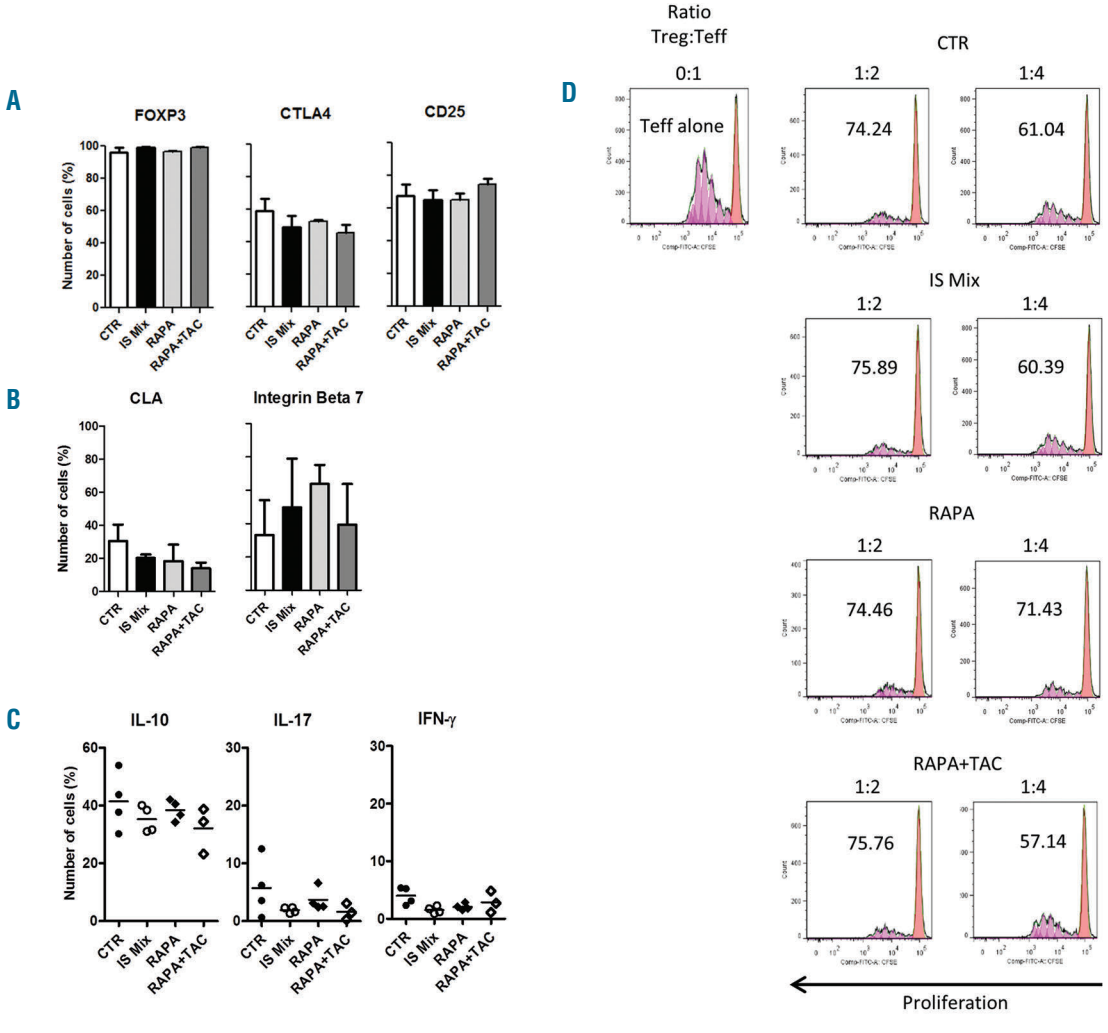

Figure 7. Effect of immunosuppressants on Treg phenotype and function in a humanized mouse model. (A) Percentages of $FOXP3^+$, $CTLA^+$ and $CD25^+$ Tregs recovered from mice receiving immunosuppressive treatment (IS Mix, RAPA and $RATA^+TAC$) and controls (CTR). (B) Percentages of CLA^+ and Integrin-$\beta7^+$ Tregs in the 4 groups of mice. (C) Percentages of Tregs producing IL-17, IFN-γ and IL-10, respectively, in the 4 groups of animals. (D) Representative histograms showing the suppressive ability of Tregs recovered from mice under immunosuppressive treatments and controls. Treg:Teff ratio 1:2 and 1:4. Data are representative of 4 independent experiments.

of alloantigen was facilitated by the presence of MPA.[42] *In vitro* studies further confirmed the pro-tolerogenic effect of MPA on the generation of Tregs[43] and on the enhancement of FOXP3 expression and co-inhibitory molecules, such as PD-1 and CTLA-4 in total $CD4^+$ T cells.[44]

The treatment with mPr alone *in vitro* or *in vivo* was not harmful for Tregs. Steroids such as dexamethasone and prednisolone have been used for decades as a basis for the treatment of inflammatory diseases and many authors have described positive effects on the maturation and expansion of Tregs in autoimmune diseases. Glucocorticoids have been suggested to amplify the IL-2-dependent expansion of $FOXP3^+CD4^+CD25^+$ T cells *in vivo*,[45] increase FOXP3 expression by Tregs in patients affected by asthma,[14] and restore the impaired suppressive function of Tregs in patients with relapsing multiple sclerosis.[46] Altogether, steroids may have a beneficial effect on Tregs by influencing other cells and altering the inflammatory environment. This idea was suggested by some studies reporting that glucocorticoids negatively control both Th1- and Th17-polarization in mice and humans. Data showed that this treatment can affect the production of Th1 or Th17 polarizing cytokines produced by innate immune cells, as well as T-cell capability to respond to these cytokines.[47,48] Furthermore, recent *in vitro* and *in vivo* studies showed that glucocorticoids can favor the establishment of pro-tolerogenic conditions. Human DCs treated with glucocorticoids up-regulate the GC-Induced Leucine Zipper protein (GILZ) and differentiate into tolerogenic cells capable of inducing IL-10–producing antigen-specific Tregs.[49]

Finally, our data have shown that the *in vivo* administration of RAPA maintained the survival and proliferation of adoptively transferred Tregs. In contrast, the proliferation of non-Treg cells was inhibited. Similar results were obtained in a murine model of heart transplant in which the adoptive transfer of a small number of alloantigen-specific Treg in the presence of a low dose of RAPA induced long-term survival of cardiac allografts.[50] The beneficial effect of RAPA on Tregs was also observed in a humanized-mouse model where segments of human arterial were transplanted into immunodeficient mice reconstituted with allogeneic human peripheral blood cells. In this model, the authors showed that the presence of RAPA enhanced the ability of sub-therapeutic numbers of human Tregs to prevent transplant rejection.[18]

The differential *in vivo* effect of RAPA and TAC is of interest. Although they are structurally similar and bind the same target (FK-binding protein), RAPA leaves the calcineurin pathway untouched. Our results confirmed data in literature showing that RAPA rather than CNI, maintains Treg function without decreasing the efficacy of the immunotherapy and leads to a prolonged allograft survival.[32]

Altogether the data obtained from our study suggest that ISDs do not affect the key functions of *ex vivo* expanded Tregs. However, the treatment can have detrimental effects on their viability and proliferative capacity. It has been reported that the percentage of circulating Tregs decreases in the months after transplantation in patients on immunosuppression with calcineurin inhibitors.[35,36] Similarly, a clinical study showed that the conversion of kidney transplant recipients from TAC+MPA treatment to RAPA mono-therapy, significantly increased the percentage and absolute number of circulating Tregs.[30] Therefore, our results confirm that TAC and MPA may have an unhelpful effect, and suggest that more tolerance-permissive agents such as RAPA should be used as adjunctive therapy with Tregs.

Finally, our data indicate that the use of a concurrent immunosuppression treatment can be compatible with adoptive Treg therapy. However, the choice of specific drugs, as well as their timing and dosing, could be an essential component of strategies to induce and maintain tolerance mediated by Treg therapy.

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